Floundering about at cell membranes: a structural view of phospholipid signaling

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Structures are now available for the majority of the enzyme families involved in the phosphorylation, dephosphorylation and hydrolysis of signaling phospholipids. Lipid kinase and phosphatase structures recapitulate catalytic motifs involved in protein phosphorylation and dephosphorylation, whereas cytosolic phospholipase A_2 manifests novel catalytic geometry. Structures have been determined for most known intracellular phospholipid 'receptor' domains, both those that bind membrane-embedded phospholipids and those that bind lipid monomers.

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Abbreviations

5-PO_4ase	inositol polyphosphate 5-phosphatase
AA	arachidonic acid
C1	protein kinase C homology-1
C2	protein kinase C homology-2
$cPLA_2$	cytosolic phospholipase A_2
DGPP	diacylglycerol pyrophosphate
FERM	four-point-one-Ezrin-Radixin-Moesin
FYVE	Fab1-YOTP-Vac1-EEA1
MAPK	mitogen-activated protein kinase
PC	phosphatidylcholine
PCTP	phosphatidylcholine transfer protein
PDB	Protein Data Bank
PE	phosphatidylethanolamine
PEBP	phosphatidylethanolamine-binding protein
PH	pleckstrin homology
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PI4K	phosphoinositide 4-kinase
PI3P	phosphatidylinositol 3-phosphate
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PIPK	phosphatidylinositol phosphate kinase
PITP	phosphatidylinositol transfer protein
PKA	protein kinase A
PLC	phospholipase C
PLD	phospholipase D
PS	phosphatidylserine
PTPase	protein tyrosine phosphatase
$sPLA_2$	secretory phospholipase A_2
START	steroidogenic acute regulatory protein-related lipid transfer

Introduction

If one were to judge by the sheer number of receptors to which they are coupled, the phospholipids and their metabolites would be arguably the most important of all second messengers (Figure 1). What is so special about phospholipids and phospholipid signaling proteins? The phosphoinositides and other signal-transducing phospholipids are compartmentalized in distinct pools in the various membranes of the cell, separated by aqueous phase barriers. Most of the proteins involved are amphitropic, meaning they can exist as either membrane or soluble proteins. Reversible translocation between the cytosol and different cell membranes is a central feature of their cell biology. The activities of the proteins feature the full range of complexities of interfacial enzymology. Their evolution reflects the unique requirements of life at an interface — hence the 'floundering about' of the title. In this review, we focus on new developments in their biophysics and structural biology. These fields are both fascinating and confounding because of the interactions of the proteins with the complex and dynamic milieu of the phospholipid bilayer.

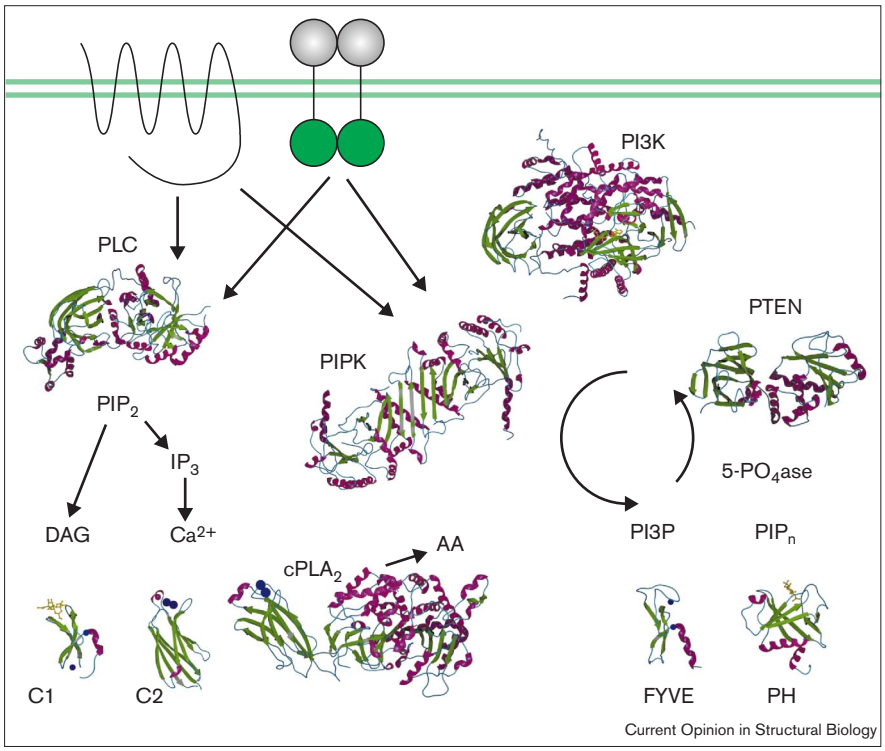
Phospholipases: the cutting edge

Phospholipases selectively hydrolyze phospholipids and are classified according to the bond that is cleaved: A_1, A_2, C and D. The hydrolysis products of phospholipases include many of the most critical second messengers in cell signaling. A now classic body of early structural work focused on the structures and mechanisms of the small secretory phospholipases A_2 ($sPLA_2$s) and, subsequently, phospholipase C (PLC). Recent attention has focused on the cytosolic phospholipases A_2 ($cPLA_2$s) and phospholipase D (PLD).

Cytosolic phospholipase A_2

$cPLA_2$ is one of the most intensively studied of all lipid signaling enzymes [1]. $cPLA_2$ is responsible for the production of arachidonic acid (AA), the precursor of numerous signaling lipids active in the inflammatory process. $cPLA_2$ is activated by Ca^{2+} and mitogen-activated protein kinase (MAPK) phosphorylation, and cleaves AA selectively from the *sn*-2 position of phospholipids. $cPLA_2$ consists of two domains, an N-terminal Ca^{2+}-dependent lipid-binding protein kinase C homology-2 (C2) domain and a C-terminal catalytic domain. The crystal structure of intact $cPLA_2$, which was determined last year, was a major advance in understanding its regulation, interfacial activation and catalytic mechanism [2••]. The active site of the catalytic domain is found in a deep hydrophobic funnel. The active site contains an unusual Ser-Asp dyad, together with a critical arginine whose precise role is elusive. A flexible lid covers the active site and its displacement upon lipid binding is postulated to explain the interfacial activation of this enzyme. In contrast to PLC-δ1 [3,4], the C2 and catalytic domains are connected by a flexible loop and lack extensive contacts (Figure 2). These features suggest that some interdomain

Figure 1



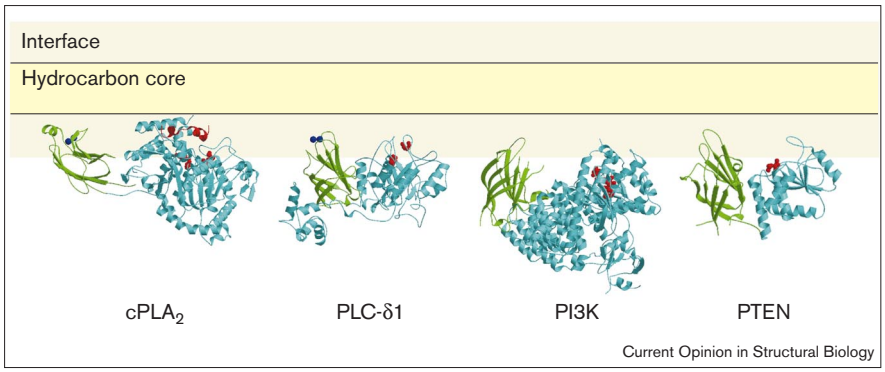
A highly simplified schematic of some of the most intensively studied pathways in phosphoinositide signaling. The receptors at the top represent generic G-protein-coupled receptors and tyrosine-kinase-linked receptors. The figure is intended to emphasize that structures are now known for representatives of nearly all of the best-known players in phosphoinositide hydrolysis, phosphorylation and dephosphorylation, as well as for the key 'receptor' domains in their downstream effectors. The structures were generated with Spock from the following PDB codes: PLC, 1QAT; PI3K, 1QMM; PIPK, 1BO1; PTEN, 1D5R; C1 domain, 1PTR; C2 domain, 1QAT; cPLA2, 1CJY; FYVE domain, 1VFY; PH domain, 1MAI. β Strands are green, helices magenta, metal ions blue and lipid headgroups yellow. DAG, diacylglycerol; IP_3, inositol (1,4,5)-trisphosphate; PIP_n, phosphatidylinositol polyphosphate.

rotation occurs to optimize the interaction between the enzyme and the membrane.

Phospholipase D: 'D' is for difficult

PLD catalyzes the breakdown of phosphatidylcholine (PC) to phosphatidic acid (PA), and plays important, albeit vaguely defined, roles in signaling and membrane trafficking. Eukaryotic PLD sequences contain two repeats of the catalytic motif $HXKX_4DX_6GSXN$, whereas bacterial PLDs and a wide range of homologous phosphodiesterases contain a single copy of the motif. Eukaryotic PLDs have been extraordinarily resistant to structural studies so far, but the crystal structure [5•] and mechanistic enzymology of Nuc, an endonuclease from *Salmonella typhimurium*, suggested a two-histidine catalytic mechanism. In this mechanism, one histidine from a symmetry related pair forms a covalent phosphoenzyme intermediate, whereas the second acts as a general acid. The Nuc structure reveals a dimer that juxtaposes two copies of the $HXKX_4DX_6GSXN$ motif responsible for catalysis in human PLD [6]. The histidine from one motif is postulated to be the catalytic nucleophile, whereas the histidine from the second copy of the motif is postulated to act as a general acid that cleaves the phosphoenzyme

Figure 2



The C2 domain (green) as 'domain of the year'. C2-domain-containing enzymes are shown in hypothetical bilayer-docked modes. Ca^{2+} ions are colored blue and selected active site residues and ligands are colored red. The C2 domain of $cPLA_2$ (PDB code 1CJY) is linked by a flexible tether and, therefore, the catalytic and C2 domains have considerable freedom to reorient themselves relative to each other. The lid of the catalytic domain is colored blue. PLC-δ1 (PDB code 1QAT) is docked with a higher confidence level than the other three structures because the structure of the polar product [inositol (1,4,5)-trisphosphate, IP_3] complex is known [3]. Membrane docking modes shown for PI3K (PDB code 1QMM) and PTEN (PDB code 1D5R) are the least certain.

intermediate formed with the first histidine. In metazoan PLDs, the two repeats of the catalytic motif presumably form an 'intramolecular dimer' resembling the arrangement seen in Nuc.

Phospholipid kinases: flattened faces, C2 domains and activation loops

We now know that phosphatidylinositol (PI) exists biologically in derivatives that are phosphorylated singly at the D-3, D-4 and D-5 positions, and in all possible combinations [7]. Each of these seven species has a distinct biological role in metazoan signaling networks. Their formation and interconversion depend on the action of specific phosphoinositide kinases and phosphatases. The regulation of phosphoinositide kinases has been studied intensively because of their close coupling to many cell surface receptors. Nevertheless, until two years ago, almost nothing was known about their three-dimensional structure. The past two years have seen the determination of the first crystal structures for archetypal members of both major families of phosphoinositide kinases: phosphoinositide 3-kinase and 4-kinase (PI3K/PI4K) [7], and the phosphatidylinositol phosphate kinases (PIPKs) [8]. Another important superfamily of signal-transducing lipid kinases is responsible for phosphorylation of diacylglycerol and sphingosine. As yet, nothing is known about their structure and they will not be discussed further in this review.

The structures of PIPK-IIβ [9] and PI3K-γ [10••] reveal that both are closely related to members of the eukaryotic protein kinase superfamily (Figure 3). There are, however, some important differences in detail. For example, PI3K-γ lacks a glycine-rich loop and substitutes the Gly•ATP phosphate interaction seen in the protein kinases with a serine. The protein and lipid kinases exemplify a theme of shared architecture and mechanism in protein and lipid phosphorylation that is also seen for dephosphorylation, as described below. It is intriguing that the phosphoinositide kinases appear to have evolved from protein kinases, rather than from any of a number of structurally unrelated small-molecule kinase families. Two relevant commonalities are a role in signal transduction and activity against large substrates. Although individual phosphoinositide molecules are much smaller than proteins, the phosphoinositide kinases are active only against membrane-embedded substrates. The substrate is, from the point of view of access by the enzyme, the bilayer. Most nucleotide, sugar and other small-molecule kinases undergo domain closure, which completely sequesters their substrates. In the case of a large protein or a membrane-embedded lipid, this is not possible.

The most interesting comparison between the protein and phosphoinositide kinases concerns the role of the activation loop. In both cases, the loop has a critical role in the enzyme's function. In the protein kinases, the conformation of the activation loop is influenced by its phosphorylation state and by binding of kinase activators, hence its name. In the phosphoinositide kinases, the principal role of the equivalent loop is determining substrate specificity. In the PI3K/PI4K family, the loop sequence determines not only the phosphoinositide substrate specificity, but also the protein kinase activity [11]. This finding also holds true for the PIPKs [12••]. The real surprise, however, is that the PIPK activation loop is also a major determinant of subcellular localization.

PIPK and PI3K use different mechanisms to interact with substrates in bilayer membranes. Both PIPK and PI3K contain an N-terminal extension to the small lobe of the catalytic domain. In PIPK, this extension forms a flattened face and, moreover, a very large platform for membrane docking [13]. In PI3K, the similar extension is not involved in dimerization nor is it poised for a membrane interaction like that seen in PIPK. Rather, a C-terminal C2 domain, of the type II topology and the non-Ca^{2+}-binding variety, appears to be the most important site for membrane docking.

Lipid phosphatases

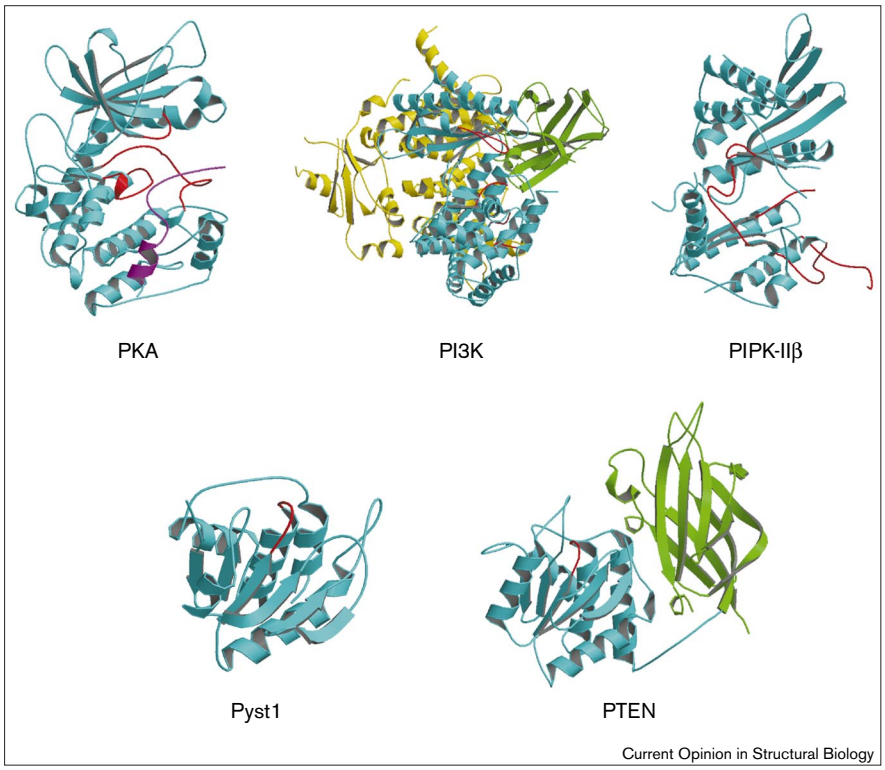
Structure of PTEN: the C2 domain strikes again

The tumor suppressor PTEN specifically removes the 3-phosphate from phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PTEN contains the signature motif HCXXGXXR(S/T), which is found in the active sites of protein tyrosine phosphatases (PTPases) and dual-specificity protein phosphatases. The crystal structure of PTEN revealed that the protein consists of two domains: an N-terminal phosphatase domain, whose similarity to PTPases had been expected, and a surprise C-terminal C2 domain [14••]. The active site of the phosphatase domain is wider than that of the PTPases in order to accommodate the phosphoinositide substrate. A tartrate ion indicates the probable position of substrate phosphate groups. The C-terminal C2 domain of PTEN lacks the acidic residues that are found in other C2 domains and are critical for binding to Ca^{2+}. It also associates with phospholipid membranes *in vitro* in the absence of Ca^{2+}. Together with the fact that the phosphatase domain and C2 domain of PTEN interact over a large surface area, the function of the PTEN C2 domain is likely to be the targeting of this enzyme to membranes.

Other lipid phosphatases

There are several other groups of lipid phosphatases for which structures have not yet been determined, although information on a homologous protein is available for at least one of these. The inositol polyphosphate 5-phosphatases have a catalytic core domain referred to as an 'inositol polyphosphate phosphatase catalytic' (IPPc) domain. Another group, Sac1 and its homologs, contains an evolutionarily conserved 300 amino acid region and has polyphosphoinositide phosphatase activity. Yet another superfamily comprises the Mg^{2+}-independent phosphatidic acid phosphohydrolase, diacylglycerol

Figure 3



Structural similarity between protein and lipid kinases and phosphatases. PDB files for the lipid kinases and phosphatases are as mentioned in captions to Figures 1 and 2, with the addition of cAMP-dependent protein kinase A (PKA) (PDB code 1APM) and dual-specificity protein phosphatase Pyst1 (PDB code 1MKP). C2 domains are colored green, active site residues and their immediate surroundings are red, catalytic domains are cyan, the inhibitory peptide bound to PKA is magenta and the remainder is yellow.

pyrophosphate (DGPP) phosphatase, sphingosine phosphate phosphatase and a number of phosphatases with nonlipid substrates. The structure of one of this last class is known, that of chloroperoxidase, and has been used as the basis for both modeling and mutational analysis of the DGPP phosphatase [15].

Phospholipid-binding and transport domains

Phospholipid monomers bind to a distinct set of binding proteins and domains that typically contain hydrophobic tunnels or deep cavities. The phosphatidylinositol transfer protein (PITP) and the phosphatidylcholine transfer protein (PCTP) are two examples of recent interest. Many of these proteins and domains have lipid transfer activity *in vitro* and are commonly referred to as lipid transfer proteins. It seems likely that many nominal lipid transfer proteins and domains have regulatory roles beyond the transport process.

The effectors of phospholipid signaling most often recognize specific lipids through conserved modular domains. These include C1 (protein kinase C homology-1), C2, FYVE (Fab1-YOTP-Vac1-EEA1), PH (pleckstrin homology), FERM (four-point-one-Ezrin-Radixin-Moesin) and vinculin tail domains, as well as small proteins such as the phosphatidylethanolamine (PE)-binding protein (PEBP). The binding of phospholipids by these domains results in the recruitment of the effector proteins to the membrane surface for action and thus the stereochemistry of lipid binding by the effector domains influences the orientation of the effectors at the membrane. These domains have been comprehensively reviewed [16] in the recent past, but there have been important new developments for the C2, FYVE and FERM domains.

Hydrophobic holes: monomer binding by lipid transfer proteins

PITPs exist in eukaryotic systems from yeast to human. The crystal structure of yeast PITP, also known as Sec14p, in complex with the detergent β-octylglucoside reveals a hydrophobic pocket large enough to accommodate the acyl chains of a phospholipid molecule [17]. Polar residues near the mouth of the pocket are postulated to confer specificity for PI molecules. Mammalian PITPs lack sequence identity to Sec14p and it remains to be seen whether they have the same overall structure and mechanism.

PCTP specifically mobilizes PC, yet its precise biological function remains unknown. Although the structure of PCTP is not known, the crystal structure of the homologous steroidogenic acute regulatory protein-related lipid transfer (START) domain of human MLN64 suggests the structure of PCTP. The crystal structure of the MLN64 START domain reveals an α+β fold that is built around a U-shaped incomplete β barrel [18•]. The most striking feature of the START domain structure is a predominantly hydrophobic tunnel extending nearly the entire length of the protein. The tunnel is large enough to

accommodate a single cholesterol or PC molecule. START domains are distinguished from most other groups of lipid transporters by their exceptionally high degree of specificity. By mapping the aligned START domain sequences onto the crystal structure, differences in the charge of tunnel wall amino acid residues have been proposed to generate lipid specificity.

Phosphatidylethanolamine-binding protein

PEBP has been proposed to play roles in membrane biogenesis, cell signaling and cell growth. Unlike PITP and the START domain, structures of PEBP show that it does not contain an internal hydrophobic tunnel or pocket [19,20]. Instead, a small cavity that has a high affinity for anions such as phosphate and PE has been observed close to the protein surface.

The C2 domain: solid support for the Ca^{2+} bridge

The best-known C2 domains bind acidic phospholipids in a Ca^{2+}-dependent manner, but the mechanism by which Ca^{2+} promotes phospholipid binding has been controversial. The crystal structure of the C2 domain of protein kinase C-α (PKC-α) in complex with a phosphatidylserine (PS) molecule reveals that the recognition of PS involves a direct interaction with one of the two bound Ca^{2+} ions [21•]. The direct bridging role for the Ca^{2+} ion in lipid binding explains the dependence on Ca^{2+}, at least for this subset of C2 domains. The orientation of the bound PS is consistent with a widely accepted model of the membrane-bound C2 domain, in which hydrophobic residues of the third Ca^{2+}-binding region (CBR3) insert into the membrane and the basic face of the domain interacts with the membrane surface.

FYVE fingers for phosphoinositides

FYVE domains are widespread modules that recognize phosphatidylinositol 3-phosphate (PI3P). The crystal structure of the Vps27 FYVE domain modeled the lipid-headgroup-binding site at the hydrophobic tip of the domain on the basis of the position of carboxylate groups from a neighboring FYVE domain in the crystal lattice [22••]. A different binding mode was suggested by a recently determined crystal structure of a dimer of tandem FYVE and VHS (Vps27p, Hrs, STAM) domains that indicated that the hydrophobic tip is part of a dimer interface and therefore, at least in the context of the dimer, cannot insert into the membrane [23•]. This site includes residues from both units of the FYVE dimer, which is consistent with NMR data indicating that the functional unit for lipid binding is a dimer [24•]. The structure of the dimer leads to a different model for membrane interaction, in which the FYVE domain is horizontal to the membrane and its apical end is involved in dimerization.

Regulation of the cytoskeleton by phospholipids: the FERM domain and the vinculin tail

The cytoskeleton is a major target for regulation by phosphatidylinositol (4,5)-bisphosphate (PIP_2). The recently determined crystal structure of a module found in membrane–cytoskeletal linkers, the FERM domain, revealed the unexpected presence of a subdomain that shares the PH fold, suggesting that the binding of phosphoinositides to the FERM domain may be through this subdomain [25••]. A parallel story has emerged from the structure of the vinculin tail [26••]. This structure revealed an unexpected similarity to another class of known lipid-binding proteins, the exchangeable apolipoproteins. For both the FERM and vinculin tail structures, new mechanisms have emerged for allosteric regulation of actin binding by phospholipids.

Conclusions

We now know the structures of representative members of most of the important protein and domain families in phospholipid signaling. Two major challenges remain. First, the dynamic nature of phospholipid membranes prevents high-resolution crystallographic or solution NMR structure determination of protein–membrane complexes. Alternative structural approaches have made major contributions, but each has its own disadvantages. These approaches are either indirect (mutagenic structure/function studies), low resolution (fluorescence, solution NMR chemical shift perturbations) or laborious in that they provide information on a one-site-per-experiment basis (electron paramagnetic resonance, solid-state NMR). Creative new experiments [27] and computational methods offer some promise, but a real breakthrough in this area seems unlikely in the near future. Second, the cell biology of phospholipid signaling remains dauntingly complex. Structure-based manipulation of phospholipid signaling proteins has already led to important insights into different aspects of signal transduction, for example, the signaling specificity of lipid kinases [11,12••], but the full potential of combined structural and cellular approaches has yet to be realized.

Update

A new structure of a bacterial PLD [28•] confirms the model for monomeric PLDs proposed on the basis of the dimeric endonuclease Nuc [5•]. The same conclusions almost certainly apply to the catalytic cores of the much larger eukaryotic PLDs. A recent sequence analysis of the inositol polyphosphate 5-phosphatases and Mg^{2+}-dependent endonucleases [29•] strongly suggests that these enzymes belong to a single superfamily whose fold is defined by the crystal structures of DNaseI and two apurinic/apyrimidinic endonucleases. The Mg^{2+}-dependent catalytic mechanism is likely to be similar for all of these enzymes. New structures of two PH domains that bind 3-phosphoinositides with high affinity have been reported [30•,31•].

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The structure of the PH domain of Grp1 alone and in complex with Ins(1,3,4,5)P_4 is reported here and in [31•]. These structures reveal an unanticipated role for a β-hairpin insertion between β6 and β7 in the direct interaction with the PIP_3 headgroup.

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• Skolnik EY, Lemmon MA: **Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology domains.** *Mol Cell* 2000, **6**:373-384.
A comparison between the structure of the PIP_3-specific PH domain of Grp1 and the PIP_3- and PIP_2-bispecific PH domain of DAPP1/PHISH sheds new light on the origins of phosphoinositide specificity. The Grp1-PH complex has additional interactions with the 5-phosphate, whereas the DAPP1/PHISH-PH complex shows more interactions with the 4-phosphate.